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(71) Applicant *(for all designated States except US)*: CHR. HANSEN A/S [DK/DK]; Bøge Allé 10–12, DK–2970 Hørsholm (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KRINGELUM, Børge [DK/DK]; Vaarbuen 48, DK–2750 Ballerup (DK). KRAGELUND, Lene [DK/DK]; Carl Plougsvej 8, 4. tv., DK–1913 Frederiksberg C (DK).

(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annae Plads 11, P.O. Box 3007, DK–1021 Copenhagen K (DK).

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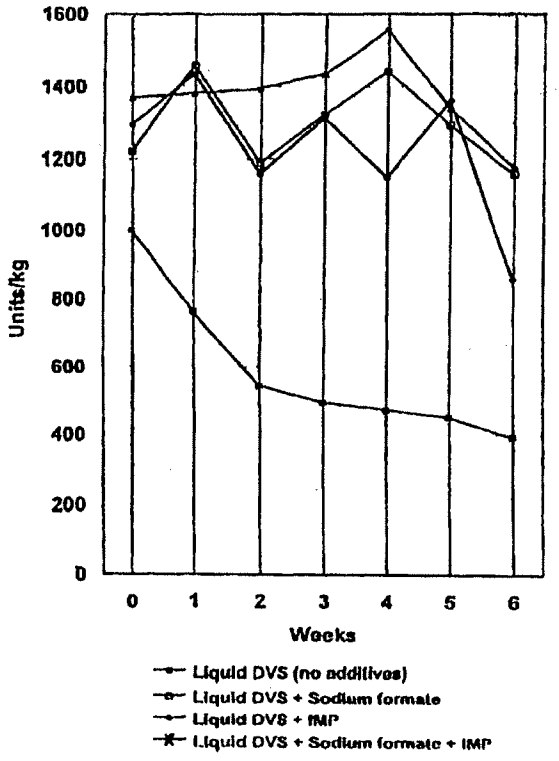
(54) Title: LIQUID STARTER CULTURES HAVING IMPROVED STORAGE STABILITY AND USE THEREOF

(57) Abstract

Liquid microbial starter culture that retains its initial metabolic activity during storage for extended periods of time. Such liquid starter cultures are useful in the manufacturing of food and feed products. Starter cultures of the invention include culture of lactic acid bacteria, e.g. *Lactococcus* species.



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LIQUID STARTER CULTURES HAVING IMPROVED STORAGE STABILITY AND USE THEREOF

FIELD OF INVENTION

The present invention relates to the field of microbial starter cultures and in particular there are provided liquid starter cultures that retain their initial metabolic activity during storage for extended periods of time. Such liquid starter cultures are useful in the manufacturing of food and feed products.

TECHNICAL BACKGROUND

Microorganisms are involved in the manufacture of food and feed products including most dairy products. Thus, bacterial cultures, in particular cultures of bacteria that are generally classified as lactic acid bacteria are essential in the making of all fermented milk products, cheese and butter. Cultures of such bacteria are referred to as starter cultures and they impart specific features to various dairy products by performing a number of functions.

Commercial dairy starter cultures are generally composed of lactic acid and citric acid-fermenting lactic acid bacteria. In the present context, the expression "lactic acid bacteria" designates a group of Gram positive, catalase negative, non-motile, microaerophilic or anaerobic bacteria which ferment sugar with the production of acids including lactic acid as the predominantly produced acid, acetic acid, formic acid and propionic acid. The industrially most useful lactic acid bacteria are found among *Lactococcus* species, *Streptococcus* species, *Enterococcus* species, *Lactobacillus* species, *Leuconostoc* species and *Pediococcus* species.

Commonly used dairy starter culture strains of lactic acid bacteria are generally divided into mesophilic organisms having optimum growth temperatures at about 30°C and thermophilic organisms having optimum growth temperatures in the range of about 40 to about 45°C. Typical organisms belonging to the mesophilic group include *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Pediococcus pentosaceus*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Lactobacillus casei* subsp. *casei*. Thermophilic lactic acid bacterial

species include as examples *Streptococcus thermophilus*, *Enterococcus faecium*, *Lactobacillus lactis*, *Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*.

Also the strict anaerobic bacteria belonging to the genus *Bifidobacterium* including *Bifidobacterium bifidum* and *Bifidobacterium longum* are commonly used as dairy starter cultures and are generally included in the group of lactic acid bacteria. Additionally, species of *Propionibacterium* are used as dairy starter cultures, in particular in the manufacture of cheese.

Additionally, organisms belonging to the *Brevibacterium* genus are commonly used as food starter cultures.

Another group of microbial starter cultures is fungal cultures, including yeast cultures and cultures of filamentous fungi, which are particularly used in the manufacture of certain types of cheese and beverage. Examples of currently used cultures of fungi include *Penicillium roqueforti*, *Penicillium candidum, Geotrichum candidum*, *Torula kefir*, *Saccharomyces kefir* and *Saccharomyces cerevisiae*.

Presently, commercial starter cultures are commonly distributed as frozen concentrates. Under these conditions, the viability of the cultures is preserved for extended periods of time and the cultures can be inoculated directly into milk without intermediate transfer. Such cultures are generally referred to as direct vat set (DVS)-cultures. Another presentation of commercial DVS-starter cultures is as freeze-dried or lyophilised cultures in the form of a powder. In this form, the starter can be shipped without refrigeration, but storage below freezing temperature is recommended.

Although commercial starters thus are available as cultures, which can be added directly to milk without any intermediate transfer or propagation, it is not uncommon that dairies produce in-house bulk starters at regular intervals depending on the requirement. A "bulk starter" is defined herein as a starter culture propagated at the dairy plant for inoculation into milk. Such bulk starters are generally made by inoculating heat treated milk with a volume of a previous bulk starter or with a freeze-dried or frozen starter culture preparation, followed by incubating the thus inoculated milk under conditions permitting

the starter culture strain(s) to propagate for a sufficient period of time to provide a desired cell number. The incubation period is typically in the range of 4 to 24 hours.

However, the preparation of such bulk starter cultures is labour intensive and it occupies much space and equipment, and there is a considerable risk of contamination with spoilage bacteria and/or phages during the step of propagation.

The use of commercial liquid starter cultures in the food and feed manufacturing industry including the dairy industry has been suggested as a useful alternative to the use of commercial frozen and freeze-dried starter cultures. The advantages for the industry by having such liquid starter cultures at its disposal would be several. Thus, it would be highly convenient and much less labour consuming to handle such starter cultures at food and feed manufacturing plants as compared to the use of the conventional frozen or freeze-dried cultures. Thus, when using liquid starter cultures, the inoculation of the material to be inoculated can be made directly e.g. by connecting the container with the liquid culture directly to the process line, thus avoiding the tedious work connected with opening several packagings of culture prior to inoculation. Additionally, it can be avoided to open the process line, as it is required when using frozen or freeze-dried cultures, which reduces the risk of contamination.

However, the use of commercial liquid starter cultures has so far not been feasible or possible, as such cultures, even if the cells of the cultures keep their viability, rapidly loose their metabolic activity such as e.g. their acid-producing (acidification) activity when kept stored even for shorter periods of time. To be commercially useful, liquid starter cultures should preferably retain their metabolic activity for at least 1 week and more, preferably for at least 2-3 weeks. Up till now it has not been possible to provide commercial liquid starter cultures having such a high stability.

It is therefore an important objective of the present invention to provide liquid starter cultures which show a high degree of storage stability in respect of retaining the metabolic activity when kept under cool storage conditions for extended periods of time.

SUMMARY OF THE INVENTION

Accordingly, it is the primary objective of the invention to provide commercial liquid microbial starter cultures for the manufacturing of food and feed products, which cultures can be stored at the site of food or feed manufacturing such as a dairy plant for extended periods of time without significant loss of their initial metabolic activity.

Thus, in a first aspect, the invention pertains to a liquid starter culture comprising an effective amount of a compound that has a metabolic activity stabilising effect, said starter culture retains at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more.

In another aspect, the invention provides a liquid starter culture capable of retaining at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more, said culture comprising at least one compound selected from the group consisting of a sugar alcohol including glycerol; carbohydrates including ascorbic acid; disaccharides including sucrose and trehalose; vitamins; antioxidants; inert gases and surfactants including Tween® compounds.

In a further aspect, there is provided a method of stabilising a liquid starter culture, the method comprising adding to the culture concentrate an effective amount of a metabolic activity stabilising compound whereby at least 50% of the initial metabolic activity of the culture concentrate is retained at a temperature of -20°C or higher for 1 week or longer.

In a still further aspect, there is provided a method of providing a liquid starter culture as defined above, said method comprising adding to the culture an amount of at least one compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds, said amount being sufficient to maintain the starter culture in a liquid state at a temperature in the range of -20°C to 0°C.

In yet another aspect, the invention pertains to a method of preparing a food or a feed product said method comprising using a stabilised liquid starter culture according to the invention.

DETAILED DISCLOSURE OF THE INVENTION

It is an essential feature of the liquid starter culture which is provided herein that the starter culture can be supplied to the site of food or feed manufacturing such as a dairy plant and be stored for extended periods of time prior to use. As used herein, the expression "liquid starter culture" relates to non-frozen liquid starter cultures having a liquid phase, e.g. an aqueous phase, content that is typically in the range of 50-90% by weight.

Thus, the liquid starter culture according to the invention comprising an effective amount of at least one compound that has a metabolic activity stabilising effect, said starter culture preferably retains at least 50% of its initial metabolic activity during storage at a temperature of -20°C or higher for 1 week or more. It is, however, preferred that the liquid starter culture retains at least 60% of its initial metabolic activity, e.g. at least 70% including at least 80% such as at least 90% of its initial metabolic activity.

As used herein the term "an effective amount" relates to an amount of a metabolic activity stabilising compound, which is added to the liquid starter culture at the starter culture production site or at the dairy plant, and which is sufficient to obtain the desired stability of the culture when kept under the above conditions. The stabilising compound which is useful in the liquid starter culture according to the invention may be any compound which permits the liquid starter culture to retain its initial metabolic activity when kept for extended periods of time at a temperature above or below 0°C.

Although the acid-producing activity is exemplified herein, this invention is intended to encompass the stabilisation of any types of metabolic activities of a starter culture. Thus, the term "metabolic activity" refers to the oxygen removal activity of the starter cultures, its acid-producing activity, i.e. the production of e.g. lactic acid, acetic acid, formic acid and/or propionic acid, or its metabolite producing activity such as the production of aroma compounds such as acetaldehyde, α-acetolactate, acetoin, diacetyl and 2,3-butylene glycol (butanediol). It will be understood that the expression "initial metabolic activity" refers to the metabolic activity of the starter organism prior to storage. In addition, it will be appreciated that the "initial metabolic activity" of the liquid starter culture is determined as described in the below Examples or any other known method of determining the production of metabolites of microbial cultures. Furthermore, the expression "retaining its

initial metabolic activity" is used interchangeably with the expression "storage stability" and refers to the capability of the liquid starter culture to substantially retain its initial metabolic activity during storage or extended periods of time under appropriate conditions.

As mentioned above, one characteristic of the liquid starter culture of the invention is its capability to retain its initial metabolic activity during storage under appropriate conditions. In preferred embodiments the liquid starter culture is stored at a temperature of -20°C or higher, such as -10°C or higher, e.g. -5°C or higher, such as 0°C or higher including 5°C or higher, such as 10°C or higher.

As it is shown in the below Examples, the liquid starter culture can be stored for a considerable period of time. Thus, the liquid starter culture according to the invention may be stored under the above conditions for at least 1 week or longer, such as at least 3 weeks or longer such as at least for 4 weeks or longer, e.g. 5 weeks or longer including 6 weeks or longer such as 7 weeks or longer. In a highly convenient embodiment, the starter culture according to the invention may be stored under the above conditions for at least 8 weeks or longer, such as at least 12 weeks or longer including at least 16 weeks or longer.

The liquid starter culture according to the invention is based on the surprising finding that a liquid starter culture can retain its initial metabolic activity during storage for a considerable period of time when a compound having a stabilising effect is added to the liquid starter culture at the starter culture production site. In presently preferred embodiments, a stabilising compound which is useful in the liquid starter culture according to the invention is a compound selected from the group consisting of formic acid, a formate, inosinate (IMP), serine and a compound involved in the biosynthesis of nucleic acids, including adenosine-5'-monophosphate (AMP), guanosine-5'-monophosphate (GMP), uranosine-5'-monophosphate (UMP), cytidine-5'-monophosphate (CMP), adenine, guanine, uracil, cytosine, adenosine, guanosine, uridine, cytidine, hypoxanthine, xanthine, hypoxanthine, orotidine, thymidine, inosine and a derivative of any of such compounds.

In a preferred embodiment of the invention the liquid starter culture contains formate at an amount which is less than 10% by weight. It is, however, preferred to add the stabilising compound at an amount which is in the range of 0.015% to 9% by weight, e.g. within the

range of 0.1% to 8% by weight, such as within the range of 0.2% to 7% by weight, e.g. within the range of 0.3% to 5% by weight, such as within the range of 0.5% to 2% by weight, including within the range of 1% to 1.5% by weight.

Additionally, the liquid starter culture may contain further conventional additives including nutrients such as yeast extract, sugars and vitamins or other substances enhancing and/or stabilising the metabolic activity and/or viability of the starter culture organisms and/or one or more compounds for lowering the freezing point of the starter culture. Thus, in useful embodiments of the invention, the liquid starter culture further comprises at least one compound that has a metabolic activity stabilising effect selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween©compounds.

In certain preferred embodiments, the liquid starter culture according to the invention contains sugar alcohols such as glycerol at an amount which is within the range of 5% to 40% by weight, e.g. as within the range of 10% to 35% by weight, including the range of 15% to 20% by weight. In a further embodiment, the liquid starter culture contains disaccharides including sucrose at an amount which is within the range of 1% to 20% by weight, e.g. within the range of 5% to 15% by weight, including the range of 10% to 12% by weight. The liquid starter culture may also contain trehalose at an amount which is within the range of 0.5 M to 1.5 M, e.g. within the range of 0.7 M to 1.2 M, including the range of 0.8 M to 1 M. In useful embodiments, the liquid starter culture contains carbohydrates, vitamins and/or antioxidants, including natural antioxidants such as vitamin C (ascorbic acid) vitamin E and lecithins and chemical antioxidants such as ascorbyl palmitate, propyl-, octyl- or dodecyl-gallat, BHA (butylhydroxyanisole) and BHT (butylhydroxytoluene). Such compounds are useful in an amount within the range of 0.01% to 1% by weight, e.g. within the range of 0.05% to 0.8% by weight, including the range of 0.1% to 0.5% by weight. Surfactants including Tween©compounds, such as Tween®20, Tween®60 and Tween®80 may be added at an amount which is within the range of 0.1% to 2% by weight, e.g. within the range of 0.5% to 1.5% by weight, including the range of 0.8% to 1% by weight.

It is convenient to provide the liquid starter culture according to the invention as a starter culture concentrate both when used in food and feed production or for the production of

metabolites that are generated by the starter culture strains. Typically, such a concentrate contains the starter culture organisms as a non-concentrated fermentate of the respective starter culture strain(s) or in a concentrated form. Accordingly, the starter culture of the invention may have a content of viable cells (colony forming units, CFUs) which is at least 10^8 CFU per ml, e.g. at least 10^9 CFU per ml, such as at least 10^{10} CFU per ml including at least 10^{11} CFU per ml, e.g. at least 10^{12} CFU per ml.

It will be understood that the liquid starter culture according to the invention can be provided as a frozen or dried, such as e.g. freeze-dried or spray-dried, starter culture as the starting material for the preparation of the liquid starter culture of the invention. Thus, it may be convenient to provide the starter culture as a frozen or dried culture and to thaw and, if required, to rehydrate the starter culture.

In accordance with the invention, any starter culture organism which is of use in the food or feed industry including the dairy industry can be used. Thus, the starter culture can be selected from a lactic acid bacterial species, a *Bifidobacterium* species, a *Brevibacterium* species, a *Propionibacterium* species or a fungal species such as a *Torula* species a *Penicillium* species, a *Cryptococcus* species and a *Saccharomyces* species. Suitable cultures of the lactic acid bacterial group include commonly used strains of a *Lactococcus* species, a *Streptococcus* species, a *Lactobacillus* species include the *Lactobacillus acidophilus, Enterococcus* species, *Pediococcus* species, a *Leuconostoc* species and *Onescoccus* species. *Lactococcus* species include the widely used *Lactococcus lactis*, including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* which are commonly used in the manufacture of cheeses with a closed texture, e.g. Cheddar, Feta and cottage cheese.

It will be appreciated, that the starter culture organism can be selected from a genetically modified strain of one of the above lactic acid bacterial strains or any other starter culture strain. As used herein the expression "genetically modified bacterium" is used in the conventional meaning of that term i.e. it refers to strains obtained by subjecting a lactic acid bacterial strain to any conventionally used mutagenization treatment including treatment with a chemical mutagen such as ethanemethane sulphonate (EMS) or N-methyl-N'-nitro-N-nitroguanidine (NTG), UV light or to spontaneously occurring mutants, including classical mutagenesis. Futhermore it is possible to provide the genetically modified bacterium by random mutagenesis or by selection of spontaneously occurring

mutants, i.e. without the use of recombinant DNA-technology, it is envisaged that mutants of lactic acid bacteria can be provided by such technology including site-directed mutagenesis and PCR techniques and other *in vitro* or *in vivo* modifications of specific DNA sequences once such sequences have been identified and isolated.

As it is usual in the dairy industry, the starter culture may comprise a mixture of strains including a mixture of strains of different lactic acid bacterial species, such as e.g. a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

The selection of strains for the starter culture of the invention will depend on the particular type of fermented food or feed product to be manufactured. Thus, e.g. for cheese and butter manufacturing, mesophilic cultures of *Lactococcus* species, *Leuconostoc* species and *Lactobacillus* species are widely used, whereas for yoghurt and other fermented milk products, thermophilic strains of *Streptococcus* species and of *Lactobacillus* species are typically used.

Fungal cultures are another group of microbial starter cultures, which may be used in accordance with the invention. Fungal cultures, such as yeast cultures and cultures of filamentous fungi, are commonly used in the manufacture of certain types of cheese and beverage. Examples of currently used cultures of fungi include *Penicillium roqueforti*, *Penicillium candidum, Geotrichum candidum*, *Torula kefir, Saccharomyces kefir* and *Saccharomyces cerevisiae*.

In a further aspect, the invention provides a liquid starter culture capable of retaining at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more, said culture comprising at least one compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween®compounds. It is, however, preferred that the liquid starter culture retains at least 60% of its initial metabolic activity, e.g. at least 70% including at least 80% such as at least 90% of its initial metabolic activity. In preferred embodiments the liquid starter culture is capable of retaining its initial metabolic activity when stored at a temperature of -20°C or higher, such as -10°C or higher, e.g. -5°C or higher, such as 0°C or higher including 5°C or higher, such as 10°C or higher.

As mentioned above and as it is shown in the below Examples, the liquid starter culture can be stored for a considerable period of time. Thus, the liquid starter culture according to the invention may be stored under the above conditions for at least 1 week or longer such as at least 3 weeks or longer such as at least for 4 weeks or longer, e.g. 5 weeks or longer including 6 weeks or longer such as 7 weeks or longer. In a highly convenient embodiment, the starter culture according to the invention may be stored under the above conditions for at least 8 weeks or longer, such as at least 12 weeks or longer including at least 16 weeks or longer.

It will be understood that the metabolic activity stabilising compounds are added to the liquid starter culture at the starter culture production site or at the dairy plant, in an amount which is sufficient to obtain the desired stability of the culture and which is sufficient to maintain the starter culture in a liquid state at a temperature in the range of -20°C to 0°C. However, in certain preferred embodiments, the liquid starter culture according to the invention contains sugar alcohols such as glycerol, disaccharides including sucrose or trehalose in the amounts specified above. In useful embodiments, the liquid starter culture contains carbohydrates, vitamins and/or antioxidants as specified above or surfactants including Tween® compounds in the amounts as specified above.

As mentioned above, it is convenient to provide the liquid starter culture according to the invention as a starter culture concentrate both when used in food and feed production or for the production of metabolites that are generated by the starter culture strains. The starter culture concentrate typically has a content of viable cells (colony forming units, CFUs) which is at least 10^8 CFU per ml, e.g. at least 10^9 CFU per ml, such as at least 10^{10} CFU per ml including at least 10^{11} CFU per ml, e.g. at least 10^{12} CFU per ml.

In accordance with the invention, any of the above-mentioned starter culture organisms which is of use in the food or feed industry including the dairy industry can be used in the liquid starter culture. Furthermore, any of the above-mentioned mixed cultures may be useful in the liquid starter culture.

It is also within the scope of the invention to provide a method of stabilising a liquid starter culture, the method comprising adding to the culture concentrate an effective amount of a metabolic activity stabilising compound whereby at least 50% of the initial metabolic

activity of the culture concentrate is retained at a temperature of -20°C or higher for 1 week or longer.

It is an advantageous feature of the method according to the invention that the above liquid starter culture is stable with respect to viability and metabolic activity including acid-producing activity for an extended period of time. Evidently, this feature implies that the method is very flexible in that the liquid starter culture can be supplied to the food or feed production plant, e.g. a dairy plant, and stored until the culture is needed. Conveniently, the liquid starter culture can be added directly to the substrate material, such as milk, meat, flour dough, wine and plant materials, such as vegetables, fruits or fodder crops. Accordingly, the use of liquid starter cultures has the advantage that no propagation, i.e. no preparation of a bulk starter at the dairy plant, of the starter organisms at the food or feed production site is necessary.

When used in accordance with the above method the stabilising compound, which is e.g. selected from the group consisting of formic acid, a formate, inosinate (IMP), serine and a compound involved in the biosynthesis of nucleic acids, including adenosine-5'-monophosphate (AMP), guanosine-5'-monophosphate (GMP), uranosine-5'-monophosphate (UMP), cytidine-5'-monophosphate (CMP), adenine, guanine, uracil, cytosine, adenosine, guanosine, uridine, cytidine, hypoxanthine, xanthine, hypoxanthine, orotidine, thymidine, inosine and a derivative of any of such compounds is conveniently added to the liquid starter culture at the production site of the starter culture. In one useful embodiment of the method according to the invention, the liquid starter culture contains formate at an amount that is less than 10% by weight including the above amounts of the stabilising compound. It is, however, preferred to add the stabilising compound at an amount which is within the range of 0.015% to 9% by weight, e.g. within the range of 0.1% to 8% by weight, such as the range of 0.2% to 7% by weight, e.g. the range of 0.3% to 5% by weight, such as the range of 0.5% to 2% by weight, including the range of 1% to 1.5% by weight.

In useful embodiments, the starter culture used in the method of the invention comprises at least one further compound having a metabolic activity stabilising effect selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds. However, it will be appreciated that

the above compounds are useful when used in the above-mentioned concentrations e.g. when the liquid starter culture in the method according to the invention is kept at temperatures below 0°C.

In an advantageous and highly convenient embodiment, the starter culture used in the method according to the invention is provided as a liquid starter culture concentrate. The use of a concentrate of the starter culture organisms involves the significant advantage over a non-concentrated liquid culture that it reduces the requirement for storage facilities significantly at the food or feed production site. Such a concentrate contains the starter culture organisms in a concentrated form, typically at a content of viable organisms of 10^{10} CFU per ml or higher including at least 10^{11} CFU per ml or higher, e.g. 10^{12} CFU per ml or higher.

In further aspects, the invention relates to a method of providing a liquid starter culture which is capable of retaining at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more, said method comprising adding to the culture an amount of at least one compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds, said amount being sufficient to maintain the starter culture in a liquid state at a temperature in the range of -20°C to 0°C. It is, however, preferred that the liquid starter culture retains at least 60% of its initial metabolic activity, e.g. at least 70% including at least 80% such as at least 90% of its initial metabolic activity. In preferred embodiments the liquid starter culture is capable of retaining its initial metabolic activity when stored at a temperature of -20°C or higher, such as -10°C or higher, e.g. -5°C or higher, such as 0°C or higher including 5°C or higher, such as 10°C or higher.

It will be understood that the metabolic activity stabilising compounds are added to the liquid starter culture at the starter culture production site or at the dairy plant, in an amount which is sufficient to obtain the desired stability of the culture and which is sufficient to maintain the starter culture in a liquid state at a temperature in the range of -20°C to 0°C. However, in useful embodiments, the liquid starter culture of the method according to the invention contains the metabolic activity stabilising compounds in the above-mentioned concentrations.

It is, however, convenient to provide the liquid starter culture of the method according to the invention as a starter culture concentrate both when used in food and feed production or for the production of metabolites that are generated by the starter culture strains. The starter culture concentrate typically has a content of viable cells (colony forming units, CFUs) which is at least 10^8 CFU per ml, e.g. at least 10^9 CFU per ml, such as at least 10^{10} CFU per ml including at least 10^{11} CFU per ml, e.g. at least 10^{12} CFU per ml.

In accordance with the invention, any of the above mentioned starter culture organisms which is of use in the food or feed industry including the dairy industry can be used in the liquid starter culture. Furthermore, any of the above-mentioned mixed culture may be useful in the liquid starter culture.

In a further aspect, the invention pertains to a method of preparing a food or a feed product said method comprising using the stabilised liquid starter culture according to the invention.

In a specific embodiment the food product is a milk-based product such as cheese, yoghurt, butter or a liquid fermented milk product, such as e.g. buttermilk or drinking yoghurt. Furthermore, the food product may be selected from a meat product, a vegetable product and a beverage such as wine and beer.

Another significant application of the method according to the present invention is the use of the liquid starter cultures as so-called probiotics. By the term "probiotic" is in the present context understood a microbial culture which, when ingested in the form of viable cells by humans or animals, confers an improved health condition, e.g. by suppressing harmful micro-organisms in the gastrointestinal tract, by enhancing the immune system or by contributing to the digestion of nutrients. A typical example of such a probiotically active product is "sweet acidophilus milk".

In further embodiments, the method according to the invention is used in the production of an animal feed such as silage e.g. grass, cereal material, peas, alfalfa or sugar-beet leaf, where bacterial cultures are inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes.

Typically, the starter organisms used in the method of preparing a food and feed product is added to the starting material at a concentration in the range of 10^5 to 10^9 CFU per ml or g of the material, such as at least 10^5 CFU per ml or g of the material, including at least 10^6 CFU per ml or g of the material, such as at least 10^7 CFU per ml or g of the material, e.g. at least 10^8 CFU per ml or g of the material, including at least 10^9 CFU per ml or g of the starting material.

The invention is further illustrated in the following non-limiting examples and the drawings wherein

Fig. 1 shows the activity of a liquid form of a commercial starter culture designated R-604 (Chr. Hansen A/S, Hørsholm, Denmark) comprising mesophilic *Lactococcus lactis* strains, with and without supplementation with Na-formate and/or IMP during storage at a temperature of 0°C for 0 to 6 weeks;

Fig. 2 shows the activity of a liquid commercial starter culture designated R-603 (Chr. Hansen A/S, Hørsholm, Denmark) comprising mesophilic *Lactococcus lactis* strains, with and without supplementation with Na-formate and/or IMP during storage at a temperature of 0°C for 0 to 6 weeks;

Fig. 3 shows the activity of a liquid form of a commercial starter culture designated TH-4 (Chr. Hansen A/S, Hørsholm, Denmark) comprising a thermophilic *Streptococcus thermophilus* strain, with and without supplementation with Na-formate and/or IMP during storage at a temperature of 0°C for 0 to 6 weeks;

Fig. 4 shows the acid-producing activity of the commercial liquid starter culture TH-3 comprising a thermophilic *Streptococcus thermophilus* strain with supplementation with various compounds during storage at a temperature of -20°C for up to 16 weeks; and

Fig. 5 shows the acid-producing activity of the liquid starter cultures TH-3, YY62, CHN19 and R-604 with supplementation with various compounds during storage at a temperature of -20°C for up to 16 weeks.

EXAMPLE 1

Study of the stabilising effect of Na-formate and IMP on the storage stability of liquid lactic acid bacterial starter culture concentrates

The stabilising effect of Na-formate, IMP or cryoprotective agents on the storage stability of liquid lactic acid bacterial starter culture concentrates was studied.

1.1 Bacterial strains, media and methods

The following liquid concentrates of lactic acid bacterial strains were used in the example: the commercial R-604 and R-603 cultures comprising mesophilic *Lactococcus lactis* strains and the TH-4 culture comprising a thermophilic *Streptococcus thermophilus* strain.

The liquid starter culture concentrates were supplemented with the following compounds, respectively:

3% Na-formate
3% Inosinate (IMP)
3% Cryoprotective agents

The culture concentrate preparations were kept at a temperature of 0, 5 and 10°C, respectively, for up to 6 weeks. Starter culture concentrates without supplement were kept at a temperature of -50°C and used as a reference.

Each week samples of the starter culture concentrate preparations were inoculated in pasteurised skimmed milk or in reconstituted skimmed milk (RSM) containing 9,5% solid matter heat treated at 135°C for 8 sec. + 99°C for 30 min, and the inoculated skimmed milk and RSM were incubated under relevant temperature conditions to permit acidification of the substrate material. Samples were collected at appropriate points in time and the acidification activity was measured as described by Foldager (1994). In addition, the viable cell number (CFU) of each sample after cultivation was determined.

1.2 Results

Under the applied experimental conditions the addition of cryoprotective agents to the culture concentrate before storage had no influence on the storage stability, i.e. the metabolic activity, of the strains (data not shown). Table 1.1 summarises the general storage stability of liquid starter culture concentrate preparations.

Table 1.1 The general storage stabilising effects of Na-formate and/or IMP on liquid starter culture concentrate

Culture	Temperature	Stability
R-604	0 °C	5 to 6 weeks
	5 °C	approximately 3 weeks
	10 °C	approximately 1 week
R-603	0 °C	5 to 6 weeks
	5 °C	approximately 3 weeks
	10 °C	less than 1 week
TH-4	0 °C	approximately 3 weeks
	5 °C	approximately 3 weeks

The results of the activity measurements during storage at a temperature of 0°C are summarised in Figs. 1, 2 and 3 for the starter culture concentrate preparations of the cultures R-604, R-603 and TH-4, respectively. The activity of the frozen reference cultures has been defined as 1000 units/kg. The initial acid-producing activity of the culture concentrate preparations is above 1000 units/kg assumingly due to an activity-stimulating effect of Na-format and IMP on the microbial culture.

The results shown in Fig. 1 clearly demonstrate that strains of R-604 were capable of retaining their initial acid-producing activity during storage at a temperature of 0°C for 5 to 6 weeks. Likewise, there is only minor loss of the initial acid-producing activity of strains of the R-603-culture during storage at 0°C for 5 to 6 weeks. Fig. 3 shows that strains of the TH-4-culture are capable of being stored at 0°C for 3 weeks without loss of initial acid-producing activity. After 6 weeks of storage the loss of the initial acid-producing activity is only about 10%.

1.4 Conclusion

This Example shows that Na-formate and/or IMP has an effect on the storage stability of liquid lactic acid bacterial starter culture concentrates as the addition of the compounds to the concentrates results in that the starter cultures retain their initial acid-producing activity for about 5 to 6 weeks when kept at a temperature of 0°C.

EXAMPLE 2

The effect of storage of a stabilised liquid mesophilic starter culture on its activity in cheese making on an industrial scale

The effect of storage on the activity of a liquid starter culture was studied in a cheese making trial on an industrial scale.

1. Bacterial strains, media and methods

The following starter culture concentrates of lactic acid bacterial strains were used in this experiment: the commercial frozen F-DVS 604 p2104250 starter culture and a liquid starter culture of R-604 comprising mesophilic *Lactococcus lactis* strains. The liquid starter culture originated from the same large scale production as the frozen starter culture.

The liquid starter culture concentrate was supplemented with a 6% of a 50% solution of sodium formate and stored at 0°C for about 4 weeks.

The cheese trials were performed at Malpass, UK, in a 1.000 litres cheese vat. A standard Cheddar recipe was used for the production of the cheese (see Table 2.1). Frozen F-DVS 604 starter culture was used as a control. The trials were performed in duplicate.

From each cheese vat 4 cheeses of about 20 kg were made, which after 24 hours were placed in a storage room at 8°C or 10°C. Samples were taken after 24 hours of storage and subjected to chemical analyses, i.e. measurement of pH of the cheese and the water, fat and salt content of the cheese.

Table 2.1 Cheese making parameters and results of the cheese trial

		Vat 1 F-DVS 604		Vat 2 Liquid culture of 604	
Parameter	Standard recipe	Actual		Actual	
Repeat No.		I	II	I	II
Milk temperature	32°C	29.5	31.5	29.9	31.0
Milk pH		6.64	6.64	6.64	6.64
Milk volume	1000 l	804 kg	806 kg	806 kg	797 kg
Starter culture added	R-604	F-DVS 604	F-DVS 604	Liquid 604	Liquid 604
Starter culture added, amount	150 g	140 g	140 g	140 g	140 g
Ripening time	40 min	30 min	30 min	30 min	30 min
Rennet added		Chymax 190, 145 g	Chymax ultra, 39 g	Chymax 190, 145 g	Chymax ultra, 39 g
Rennet added, time		11:50	10:30	12:20	10:00
Set time	40 min	48 min	40 min	45 min	40 min
Cutting time		12:38	11:10	13:05	10:40
Scalding start		12:50	11:20	13:18	10:50
Scalding temp.	40.5	40.7	40.3	41.0	40.8
Scalding time	45 min	45 min	40 min	45 min	35 min
Pitch time		14:00	12:40	14:30	12:10
Rennet to pitch	2 h 15 min	2 h 10 min	2 h 10 min	2 h 10 min	2 h 10 min
Whey off, TA%		0.1	0.11	0.1	0.11
Milling, time		15:50	14:30	16:15	14:00
Mill TA%	0.45	0.43	0.41	0.46	0.45
Rennet to mill	3 h 50 min	4 h 00 min	4 h 00 min	3 h 55 min	4 h 00 min
Composition of 24 hours cheese		Fat: 33% Moisture: 36.03% Salt: 2.4% pH: 5.57	Fat: 33% Moisture: 36.7% Salt: 2.2% pH: 5.54	Fat: 34% Moisture: 37.27% Salt: 1.5% pH: 5.38	Fat: 34% Moisture: 35.99% Salt: 2.4% pH: 5.5

2.2 Results

The parameters for the cheese trial and the results are shown in Table 2.1 below. As it appears, the activity of the liquid starter culture was as good or even slightly better compared to the frozen commercial starter culture F-DVS 604. The better acidification

results when using the liquid culture are obtained eventhough the liquid cultures are slightly diluted with sodium formate, and consequently contain a correspondingly lower cell count compared to the control starter culture.

The rennet to mill were obtained in about 4 hours for both starter cultures, however the liquid starter culture had a slightly higher acidity at milling. The compositions of the cheeses produced are given in Table 2.1.

2.3 Conclusion

This industrial trial shows that the addition of sodium formate to a liquid starter culture concentrate has an effect on the storage stability of the culture as the addition of the compound to the liquid culture concentrate results in that the starter culture has retained its initial acid-producing activity for about 4 weeks when kept at a temperature of 0°C. The liquid starter culture shows the same activity after storage as commercial, frozen starter culture, and thus is very useful in the cheese industry.

EXAMPLE 3

The effect of storage of a stabilised liquid thermophilic starter culture on its activity in cheese making on an industrial scale

The purpose of this trial was to compare the activity of the liquid thermophilic starter culture DVS TH-4, which was kept at 0°C for 4-5 weeks before testing, with the frozen DVS TH-4 from the same batch fermentation (batch 2108513). The trial was carried out in Italian pizza cheese at the dairy Ambrosi S.p.A., Castenedolo, Italy.

3.1 Bacterial strains, media and methods

The following starter culture concentrates of lactic acid bacterial strains were used in this experiment: the commercial frozen F-DVS TH-4 starter culture comprising thermophilic *Streptococcus thermophilus* strains and a liquid starter culture DVS of TH-4. The liquid starter culture originated from the same large-scale production (batch 2108513) as the frozen starter culture.

The liquid starter culture concentrate was supplemented with a 6% of a 50% solution of sodium formate and stored at 0°C for about 4-5 weeks.

The starter cultures concentrate were inoculated in milk, having the following parametres:

Fat content	3.20
Protein content	3.16
Lactose	4.74
pH of the fresh milk	6.50

Citric acid was used to decrease pH of the milk before adding the starter culture and rennet. The dosage of citric acid was about 2.3 kg per 5000 litres. The citric acid was diluted in cold water and then added to the milk. pH before adding the rennet was 6.15 to 6.20.

The cheese trail was performed in a 5000 litres cheese vat using 500g starter culture for inoculation. The cheese vat was a CMT vat of 5000 litres with horizontal stirring and cutting. Stainless steel wires were used for the cutting and stirring.

The recipe used for the production of Italian pizza Mozzarella cheese (Table 3.1). Frozen F-DVS TH-4 starter culture was used as a control (batch 2108513).

For pizza Mozzarella the cheese blocks (1 kg each) are brine salted up to 1.5% salt and then vacuum packed.

Table 3.1 The recipe for the production of Italian pizza Mozzarella cheese

Filling time	30 min
Temperature of the milk	36-37°C
Citric acid	2.3 kg/5000 litres
Culture inoculation	Beginning of filling, 500g/5000 litres
Renneting time	15 min
Cutting/stirring	45 min
Draining	15 min
To cheese table	pH 6.10
Total draining	pH 5.5-5.7
Stretching at pH	pH 5.20
Stretching water temp.	80-85°C
Cheese curd temperature	53-55°C
From inoculation to stretch	2½ to 3 hours.

3.2 Results

Table 3.2 shows the fermentation for the frozen and the liquid form of the commercial starter culture designated TH-4 (Chr. Hansen A/S, Hørsholm, Denmark) comprising a thermophilic *Streptococcus thermophilus* strain. As described above, the liquid starter culture was supplemented with sodium-formate during storage at a temperature of 0°C for 4-5 weeks. From each vat whey, samples were taken and subjected to chemical analyses, i.e. measurement of fat, protein and lactose content of the cheese.

Table 3.2 Results of the cheese trial

	TH-4 liquid (500ml) vat 14		TH-4 F-DVS (500g) vat 15 (control)		TH-4 liquid (500 ml) vat 16	
	pH	Time (min)	pH	Time (min)	pH	Time (min)
Filling	6.20	0	6.20	0	6.20	0
Culture	6.20	5	6.20	5	6.20	5
Rennet	6.15	35	6.18	35	6.20	35
Cutting	6.15	50	6.15	50	6.15	55
Draining	6.10	95	6.05	90	6.00	100
Cheese table	5.95	110	6.00	105	5.86	115
pH	5.65	150	5.90	115	5.78	130
pH	5.53	160	5.70	135	5.63	135
pH	5.35	175	5.56	150	5.45	150
pH	5.26	190	5.38	165		
Stretching	5.20	195	Cooled down		Cooled down	
Whey composition	Fat 0.36 Protein 1.08 Lactose 5.08		Fat 0.40 Protein 1.10 Lactose 4.98		Fat 0.44 Protein 1.02 Lactose 5.08	

Vat no. 15 and 16 were cooled down by adding cold water to the cheese table. Due to lack of stretching capacity they had to be stretched the following day.

As it appears from Table 3.2, the activity of the liquid starter culture was as good or even a slightly better compared to the frozen commercial starter culture F-DVS TH-4.

3.3 Conclusion

This industrial trial shows that the addition of sodium formate to a liquid starter culture concentrate has an effect on the storage stability of the culture as the addition of the compound to the liquid culture concentrate results in that the starter culture has retained its initial acid-producing activity for about 4-5 weeks when kept at a temperature of 0°C. The liquid starter culture shows the same activity after storage as commercial, frozen starter culture.

EXAMPLE 4

Study of the stabilising effect of various compounds on the storage stability of liquid lactic acid bacterial starter culture concentrates

The objective of this study was to obtain a bacterial liquid starter culture, which is capable of retaining its initial acid-producing activity when stored for at least 3 month. The storage temperature in this study was -20°C in order to prolong the stability.

The stabilising effect of glycerol, sucrose, nitrogen, ascorbic acid and Tween®80 on the storage stability of a liquid lactic acid bacterial starter culture concentrate was studied.

4.1 Bacterial strains and methods

4.1.1 Bacterial strains

The following liquid culture concentrates of commercial lactic acid bacterial strains were used in this study: The TH-3 culture comprising thermophilic *S. thermophilus*, the R-604 culture comprising mesophilic *L. lactis*, the CH-N19 culture is a mixed culture of mesophilic strains and the YY62 culture comprising a mixture of 2 different *L. lactis spp lactis* strains (14.4 and 42%), *L. lactis spp cremoris* (21%), *Leoconostoc* (7.4%) *and S. thermophilus* (14.4%).

4.1.2 Storage solutions

In order to stabilise the cells during storage several compounds were tested in various combinations as shown in Table 4.1 using the cultures TH-3 (Fig. 4) and CH-N19. In the following experiments with the cultures R-604 and YY62 only the solution showing the most stabilising effect, named F4, was used (Fig. 5). The storage solutions were kept at -20°C.

Table 4.1 The storage solutions (F1-F4) used. The added compounds are shown in W/vol.

Solutions	F1	F2	F3	F4
Na formate 2%	+	+	+	+
glycerol 35%	+	+	+	+
sucrose 12%				+
head space N_2	+	+		+
ascorbic ac.0.1%	+		+	+
Tween®80 0.8%	+	+	+	+
trehalose 1M				
grindox 1%				
TH-3 conc. 52%	+	+	+	+
dilution water to 100%	+	+	+	+

4.1.3 Methods

Inoculation and activity test

100 and 200 ml bottles of reconstituted skimmed milk (RSM) containing 9,5% solid matter heat treated at 135°C for 8 sec. + 99°C for 30 min, was prepared one day in advance and kept at 5°C until use. The storage solutions of the liquid starter culture of TH-3 (kept at -20°C) and the frozen starter culture of TH-3 (stored at -50°C) were kept at 5°C before inoculation.

The milk was inoculated with 0.01% of the storage solutions F1 to F4. The inoculation was performed at 5°C and the bottles were hereafter placed in pre-warmed water bath at 37°C with the calibrated pH electrodes connected to the data logger. The pH measuring was continued for at least 16 hours, and pH after 4 hours incubation was used for comparison of samples and for calculating the acid-producing activity (in units/kg) of the samples. The pH of the samples was compared with the pH of the frozen pellets, which activity has been defined as 1000 units (as reference).

4.3. Results and discussion

Figures 4 and 5 show clearly that the addition of compounds such glycerol, sucrose, nitrogen, Tween®80 and ascorbic acid to liquid starter culture concentrates has an effect on the storage stability of the culture as the addition of the compounds to the liquid culture concentrates results in that the starter cultures have retained their initial acid-producing activity for about 2-3 months when kept at a temperature of -20°C. The liquid cultures of TH-3 and R-604 were capable of retaining their initial acid-producing activity during storage for approximately 3 months and the liquid culture of YY62 for approximately 2 months. Gas chromatography and High performance liquid chromatography (HPLC) analysis of the YY62 samples show that all the relevant components such as acetaldehyd, α-acetolactate, diacetyl, acetoin and butanediol are produced during fermentation (data not shown) showing that the metabolic activity in general is intact.

REFERENCE

Foldager, L. 1994. Determination of acidification activity in F-DVS by the LF method. Analytical Procedure Q-Ap-039.dk, Chr. Hansen A/S.

CLAIMS

1. A liquid starter culture comprising an effective amount of at least one compound that has a metabolic activity stabilising effect, said starter culture retains at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more.

2. A liquid starter culture according to claim 1, where the compound that has a metabolic activity stabilising effect is selected from the group consisting of formic acid, a formate, IMP and a compound involved in the biosynthesis of nucleic acids and a derivative of any of such compounds.

3. A liquid starter culture according to claim 2 that contains formate at an amount which is less than 10% by weight.

4. A liquid starter culture according to claim 1 or 2, further comprising a metabolic activity stabilising compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds.

5. A liquid starter culture according to claim 1 where the starter culture is provided as a starter culture concentrate.

6. A liquid starter culture according to claim 1 comprising at least 10^8 CFU of starter culture organisms.

7. A liquid starter culture according to claim 1 wherein the starter culture organism is selected from the group consisting of *Bifidobacterium* spp., *Brevibacterium* spp., *Propionibacterium* spp., *Lactococcus* spp. including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus* spp. including *Lactobacillus acidophilus*, *Streptococcus* spp., *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Onescoccus* spp. and fungal spp. including *Pencillium* spp., *Cryptococcus* spp. and *Saccharomyces* spp.

8. A liquid starter culture capable of retaining at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more, said culture comprising at least one compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds.

9. A liquid starter culture according to claim 8 where the starter culture is provided as a starter culture concentrate.

10. A liquid starter culture according to claim 8 comprising at least 10^8 CFU of starter culture organisms.

11. A liquid starter culture according to claim 8 wherein the starter culture organism is selected from the group consisting of *Bifidobacterium* spp., *Brevibacterium* spp., *Propionibacterium* spp., *Lactococcus* spp. including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus* spp. including *Lactobacillus acidophilus*, *Streptococcus* spp., *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Onescoccus* spp. and fungal spp. including *Pencillium* spp., *Cryptococcus* spp. and *Saccharomyces* spp.

12. A method of stabilising a liquid starter culture, the method comprising adding to the culture concentrate an effective amount of a metabolic activity stabilising compound whereby at least 50% of the initial metabolic activity of the culture concentrate is retained at a temperature of -20°C or higher for 1 week or longer.

13. A method according to claim 12 wherein the stabilising compound is selected from the group consisting of formic acid, a formate, IMP, a compound involved in the biosynthesis of nucleic acid and a derivative of any of such compounds.

14. A method according to claim 13 wherein the liquid starter culture contains formate at an amount which is less than 10% by weight.

15. A method according to claim 12 or 13 comprising adding at least one further compound that has a metabolic activity stabilising effect selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid,

disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds.

16. A method according to claim 12 wherein the starter culture is provided as a starter culture concentrate.

17. A method according to claim 12 wherein starter culture organism is selected from the group consisting of *Bifidobacterium* spp., *Brevibacterium* spp., *Propionibacterium* spp., *Lactococcus* spp. including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus* spp. including *Lactobacillus acidophilus, Streptococcus* spp., *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp. and fungal spp. including *Pencillium* spp., *Cryptococcus* spp. and *Saccharomyces* spp.

18. A method of providing a liquid starter culture which is capable of retaining at least 50% of its initial metabolic activity at a temperature of -20°C or higher for 1 week or more, said method comprising adding to the culture an amount of at least one compound selected from the group consisting of a sugar alcohol including glycerol, carbohydrates including ascorbic acid, disaccharides including sucrose and trehalose, vitamins, antioxidants, inert gases and surfactants including Tween® compounds, said amount being sufficient to maintain the starter culture in a liquid state at a temperature in the range of -20°C to 0°C.

19. A method according to claim 18 wherein the starter culture is provided as a starter culture concentrate.

20. A method according to claim 18 wherein starter culture organism is selected from the group consisting of *Bifidobacterium* spp., *Brevibacterium* spp., *Propionibacterium* spp., *Lactococcus* spp. including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus* spp. including *Lactobacillus acidophilus, Streptococcus* spp., *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp. and fungal spp. including *Pencillium* spp., *Cryptococcus* spp. and *Saccharomyces* spp.

21. A method of preparing a food and a feed product, said method comprising using a stabilised culture according to any of claims 1-11.

22. A method according to claim 21 wherein the food product is selected from a milk based product, a meat product, a vegetable product and a beverage.

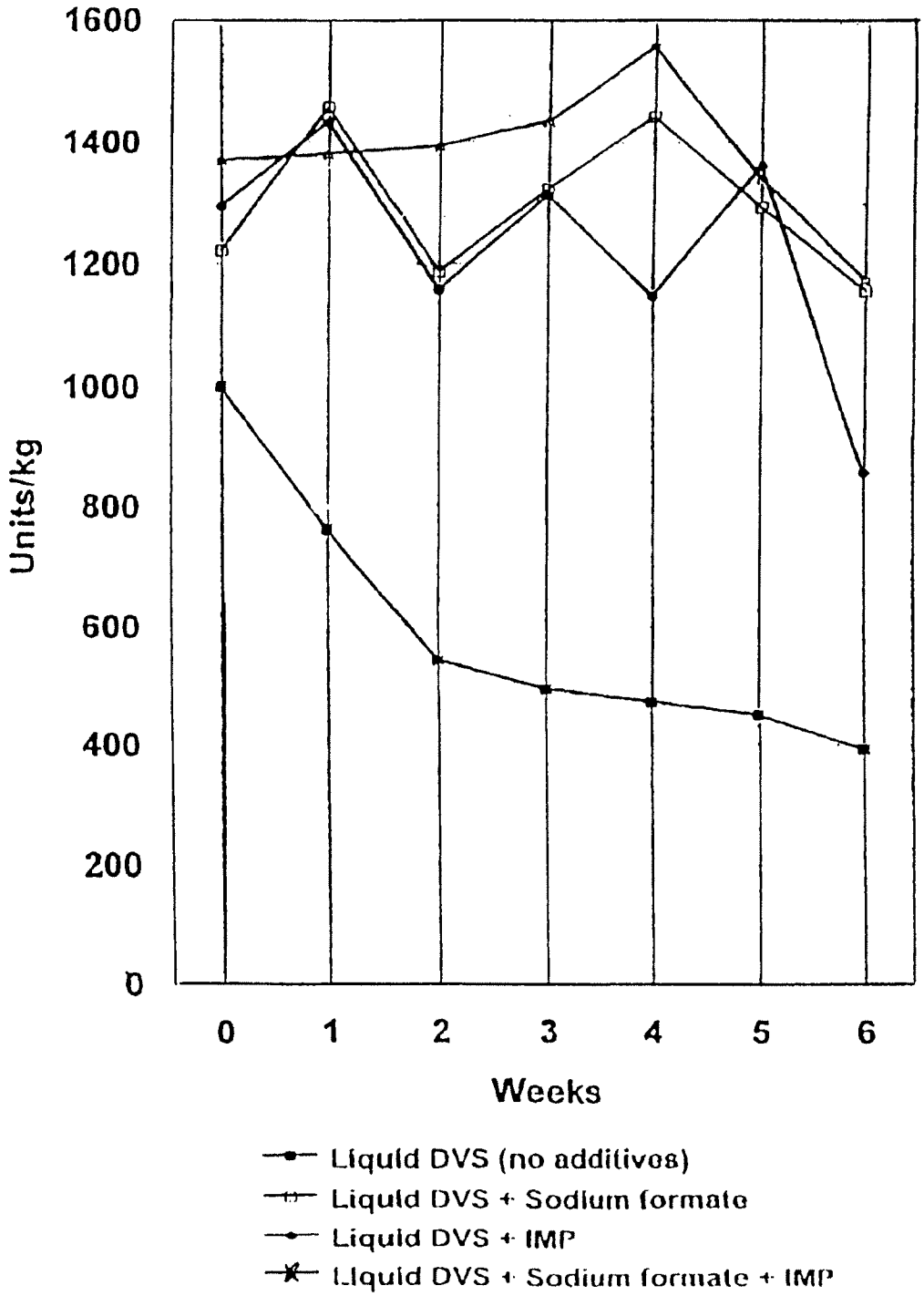

Fig. 1

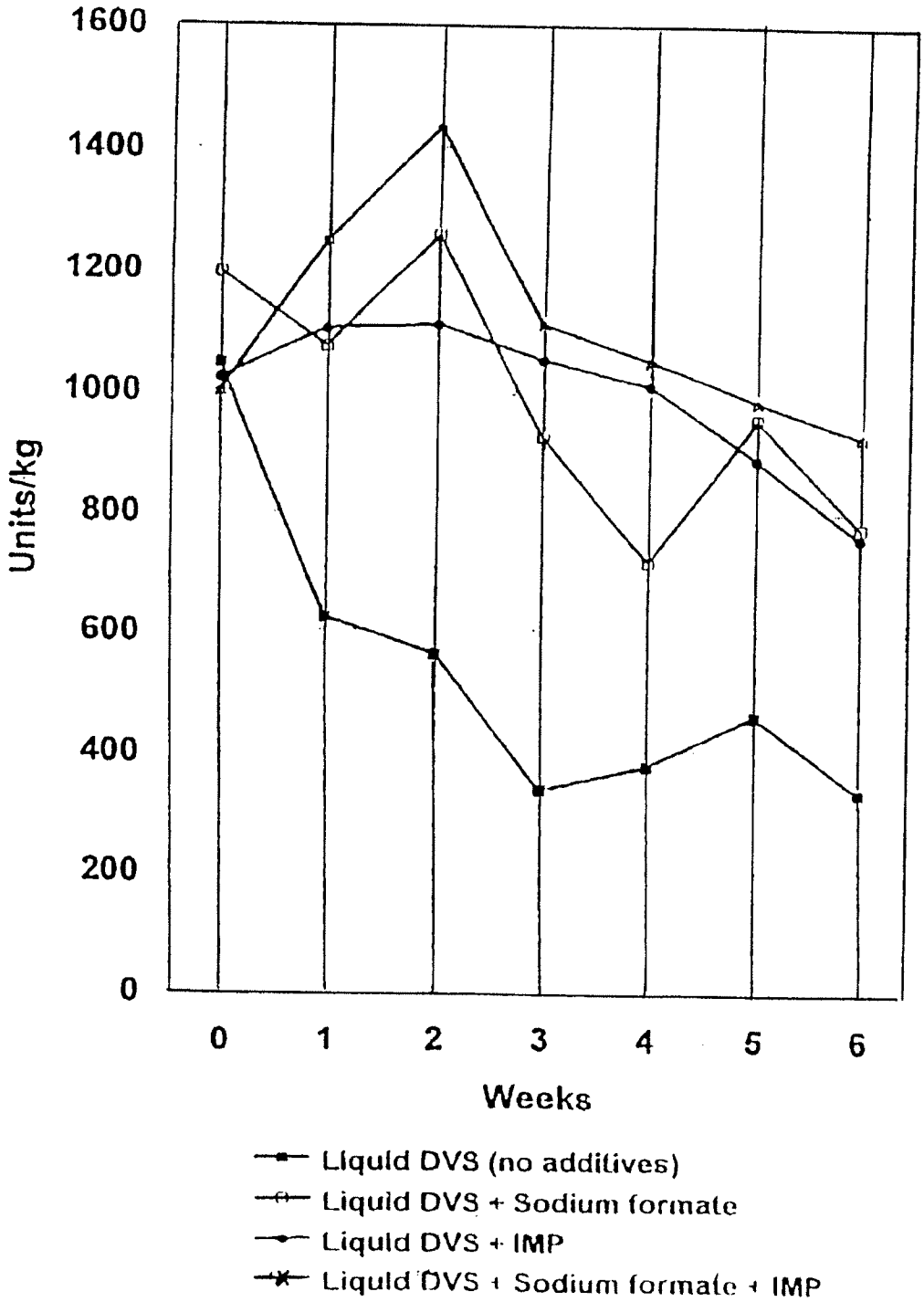

Fig. 2

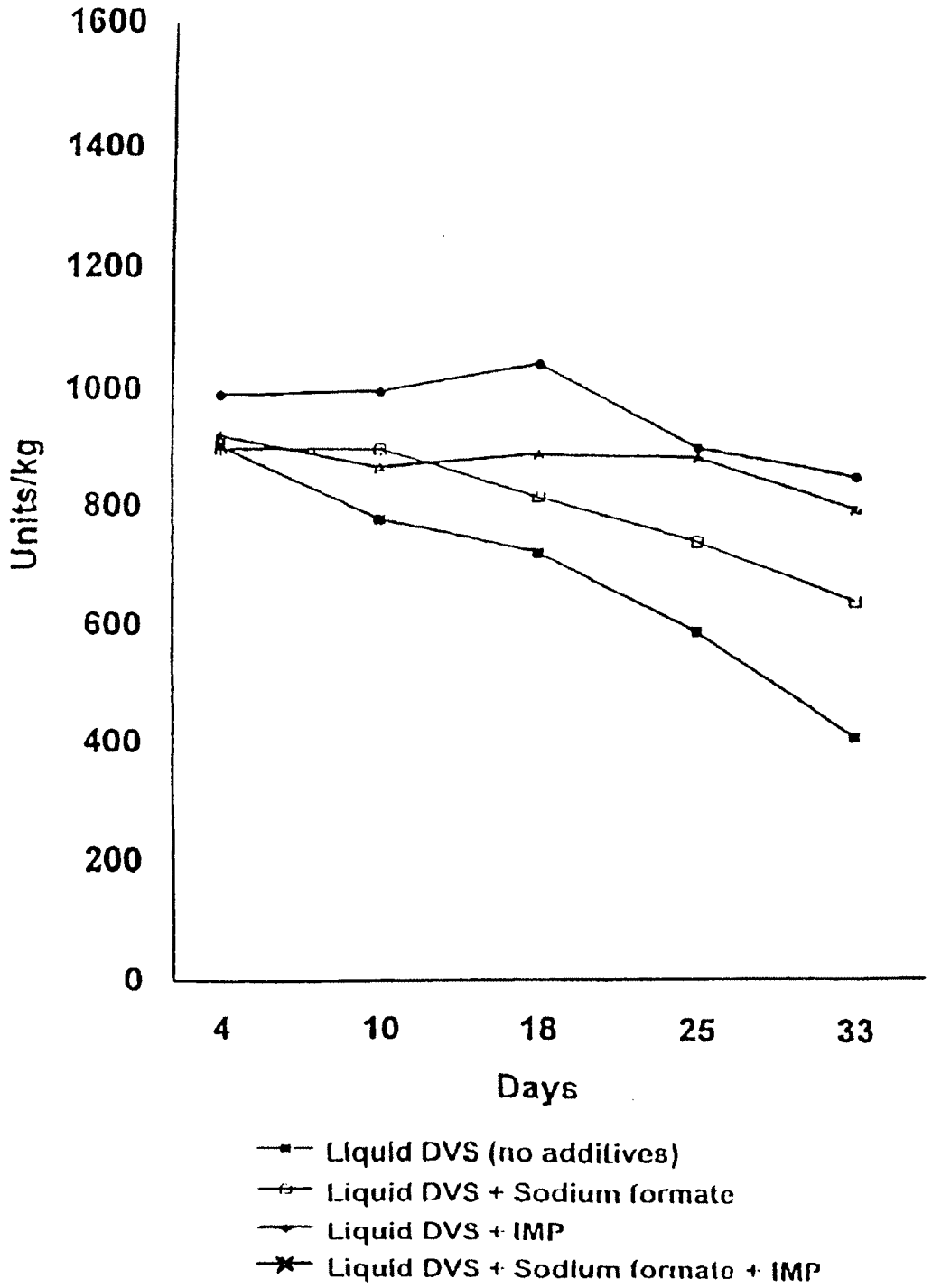

Fig. 3

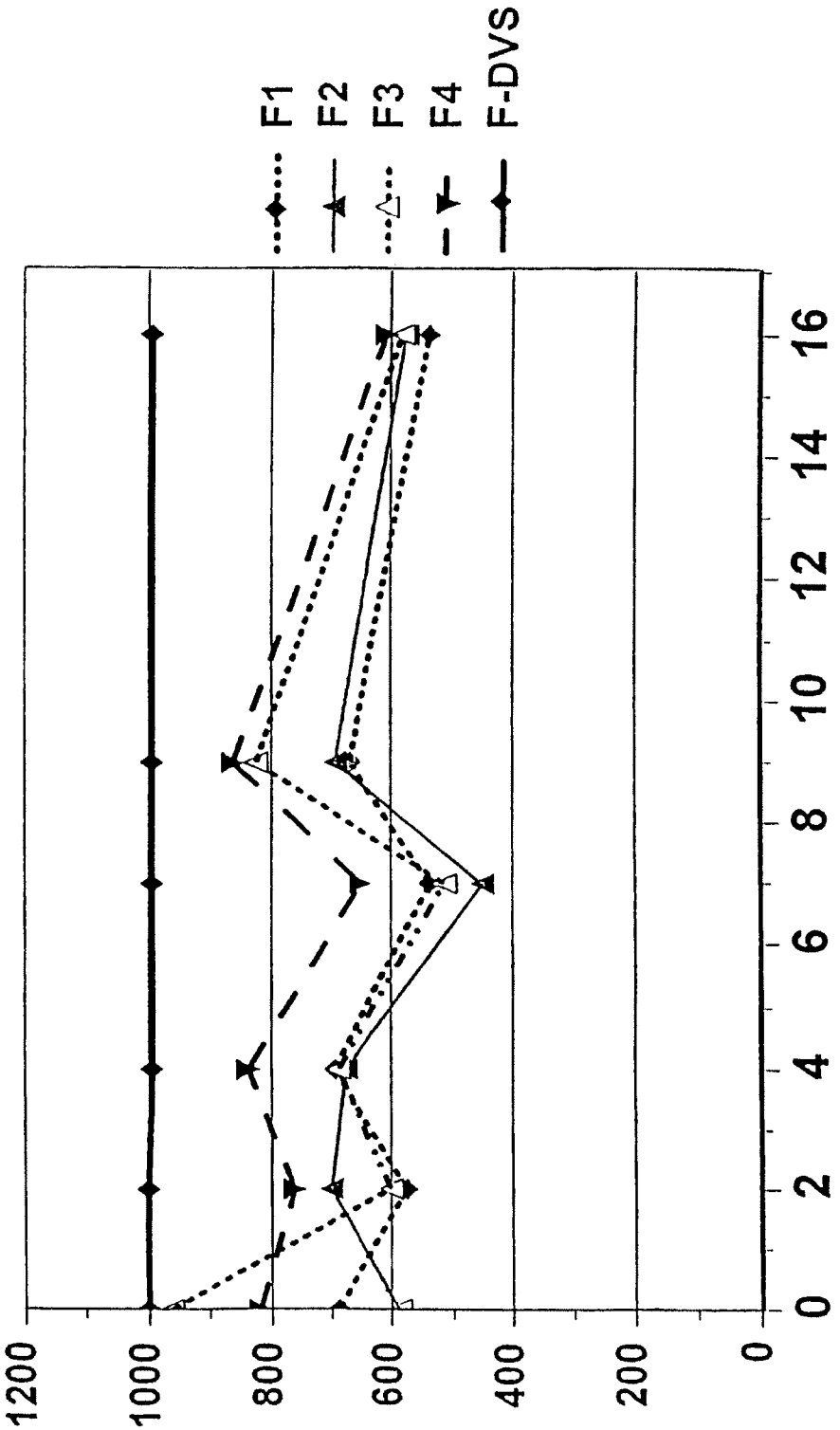

Fig. 4

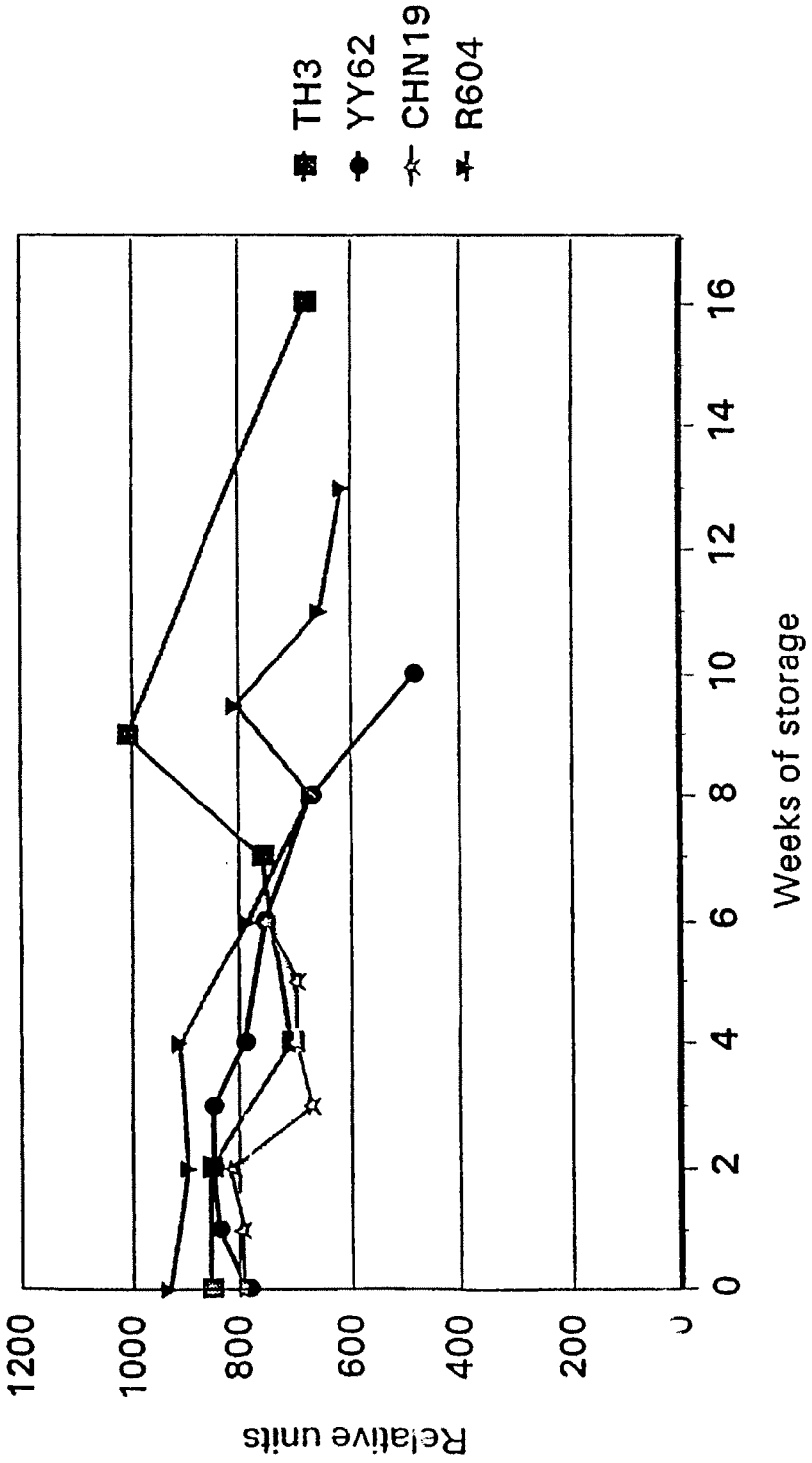

Fig. 5

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(71) Applicant *(for all designated States except US)*: CHR. HANSEN A/S [DK/DK]; Bøge Allé 10–12, DK–2970 Hørsholm (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KRINGELUM, Børge [DK/DK]; Vaarbuen 48, DK–2750 Ballerup (DK). KRAGELUND, Lene [DK/DK]; Carl Plougsvej 8, 4. tv., DK–1913 Frederiksberg C (DK).

(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Annae Plads 11, P.O. Box 3007, DK–1021 Copenhagen K (DK).

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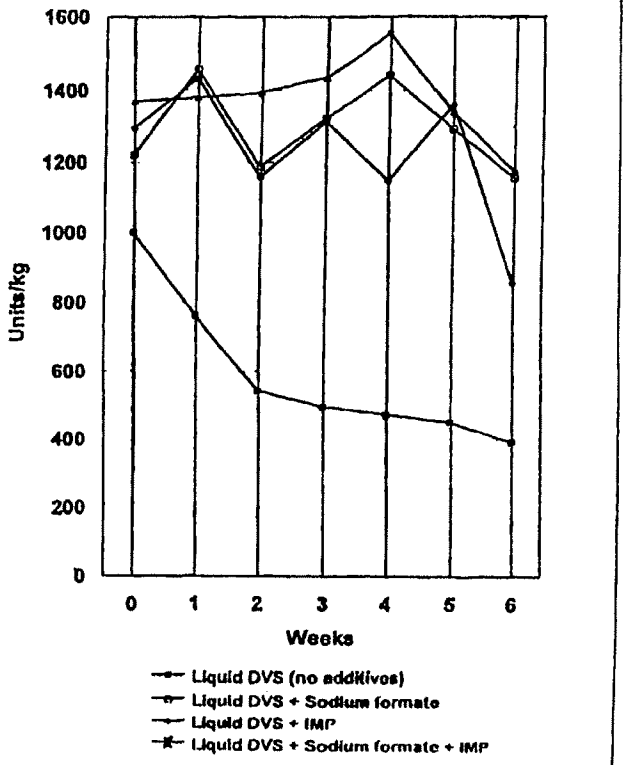
(54) Title: LIQUID STARTER CULTURES HAVING IMPROVED STORAGE STABILITY AND USE THEREOF

(57) Abstract

Liquid microbial starter culture that retains its initial metabolic activity during storage for extended periods of time. Such liquid starter cultures are useful in the manufacturing of food and feed products. Starter cultures of the invention include culture of lactic acid bacteria, e.g. *Lactococcus* species.



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INTERNATIONAL SEARCH REPORT

nal Application No
PCT/DK 99/00723

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/04 C12N1/38 A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, FSTA, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 220 548 A (CHIMICASA GMBH) 6 May 1987 (1987-05-06) page 2, paragraph 1 -page 4, paragraph 1 examples 1,3 page 10, paragraph 2 claims 1-4,7,8 ---	1-7, 12-17, 21,22
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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
31 August 2000	13.09.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

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	International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

ational application No.
PCT/DK 99/00723

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1 ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3 ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

2,3,13,14 (all completely); 1,4-12,15-22 (all partially) (inventions 1 and 3)

4 ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 2,3,13,14 (all completely); 1,4-7,12,15-17,21, 22 (all partially)

A liquid starter culture (LSC) comprising an effective amount of at least one compound that has a metabolic activity stabilising effect, where the stabilising compound is selected from the group consisting of formic acid, formate (preferably at less than 10% w/w), IMP and a compound involved in the biosynthesis of nucleic acids and derivatives of such compounds, said starter culture retaining at least 50% of its initial metabolic activity at a temperature of -20 degr. C or higher for 1 week or more. An LSC as said further comprising a compound selected from the groups listed in claim 4, or an LSC as said, preferably provided as a starter culture concentrate, or preferably comprising at least 10E8 CFU of starter culture organisms, or preferably wherein the starter culture organism is selected from the group of organisms listed in claim 7. Methods of stabilising an LSC as said, comprising adding a stabilising compound as said, and methods of preparing a food and a feed product comprising using an LSC as said.

2. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of sugar alcohols including glycerol.

3. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of carbohydrates including ascorbic acid.

4. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of disaccharides including sucrose and trehalose.

5. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of vitamins, compounds covered by invention 3 being excepted.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

6. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of antioxidants, compounds covered by invention 3 being excepted.

7. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of inert gases.

8. Claims: 1,4-12,15-22 (all partially)

As invention 1, but where the stabilising compound is selected from the group consisting of surfactants including Tween compounds.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claim 4, 8, 15 and 18, in referring to 'carbohydrates including ascorbic acid' (invention 3), relate to an extremely large number of possible compounds, such as mono-, di- and polysaccharides as well as many special categories of carbohydrates, including ascorbic acid (see, e.g., Oxford Dictionary of Biochemistry and Molecular Biology, Smith A D et al. (eds.), Oxford 1997, entry 'Carbohydrates'). Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, said claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for, and restricted to ascorbic acid, as this compound, being part of the aforementioned claims falling within invention 3, appears to be supported and disclosed, particularly in example 4.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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